A PHOSPHOLIPASE A_2 ENZYME, ANTIBODIES AND INHIBITORS THERETO

Related Application

[0001] This application claims priority to Korean Patent Application No. KR10-2002-0036249, filed June 27, 2002, which is hereby incorporated by reference in its entirety.

Field of the Invention

[0002] The present invention relates to a novel phospholipase A_2 (PLA_2) enzyme, antibodies that react with the phospholipase A_2, enzyme inhibitor and use and the preparation method thereof.

Background of the Invention

[0003] Red blood cells (RBCs) enhance platelet aggregation *in vitro* induced by calcium ionophores, collagen, thrombin, shear stress and the like. Collagen-stimulated platelets aggregate three times more effectively and discharge seven times more adenosine diphosphate (ADP) in the present of RBCs than in the absence of RBCs. This increased activity shows that RBCs are clinically correlated with pathophysiological responses of platelets.

[0004] When RBCs are stimulated by the Ca^{2+} ionophore A23187 and shear stress, they release arachidonic acid (AA) from membrane phospholipids, possibly by the action of PLA_2 from cell membrane. There are several types of PLA_2, such as $cPLA_2$ (cytosolic phospholipase A_2) existing in cytosol and $sPLA_2$ (secretory phospholipase A_2) released to cell exterior. These enzymes have been isolated from various species. Paysant *et al.* detected PLA_2 activity in RBC membrane from rat and human (Paysant M. et al.; *Bull. Soc. Chim. Biol.* **52**, pp1257-1269, 1970) and Kramer *et al.* described the purification of a calcium dependent 18.5-kDa PLA_2 from sheep RBC membrane (Kramer R. M. et al.; *Biochim. Biophy. Acta*, **507**, pp381-394, 1978). Adachi *et al.* reported detecting a calcium-independent cytosolic PLA_2 preferentially hydrolyzing phosphatidylethanolamine to phosphatidylcholine in chicken RBCs (Adachi, I. Toyoshima, S. & Osawa, T.; *Arch. Biochem. Biophys.* **226**, pp118-124, 1983). However, there has been no report concerning the antibody or inhibitor to specifically inhibit the activity this enzyme. Moreover, the RBC forms of PLA_2 have not been well studied.

[0005] The arachidonic acid released from RBCs is subsequently metabolized to eicosanoids. Eicosanoids dissociated by cyclooxygenase form prostaglandins. Prostaglandins interacting with platelets are then converted into thromboxanes. Eicosanoids dissociated by lipoxygenase form leucotrienes.

[0006] Eicosanoids play the role of messenger in various physiological functions and also take part in various phenomena such as hematopoiesis, the inflammation reaction, blood coagulation and control of blood pressure. Prostaglandins (PGs) are formed from almost all the cell in human body and stimulate blood constriction, alleviate pain resulting from an inflammation reaction and inhibit RBC volume regulation and filterability. Thromboxanes (TX) formed from platelets take part in forming thrombus comprising aggregating platelet and reduce the velocity of blood flow. Leucotrienes (LT) are formed from leucocytes and play a crucial role in the induction of inflammation and allergic responses.

[0007] From the discussion above it is clear that the eicosanoids play an indispensable regulatory role *in vivo*. However, overproduction of eicosanoids enhances platelet aggregation, results in the accumulation of thrombus, induces excessive constriction of blood vessel and finally induces various diseases such as arteriosclerosis, cerebral infarction, angina, cardiac infarction, chronic inflammation syndrome, other disorders of the immune system, cancer and the like.

Summary of the Invention

[0008] According to one aspect, the present invention provides a novel cytosolic PLA_2 enzyme isolated from RBC.

[0009] The present invention provides the antibody and inhibitor against the above enzyme.

[0010] The present invention also provides the preparation method for preparing above enzyme.

[0011] The present invention also provides a pharmaceutical composition comprising a therapeutically effective amount of antibody or inhibitor against $rPLA_2$ and a pharmaceutically acceptable carrier, for the treatment of diseases caused by disorder of hematopoietic system related to $rPLA_2$ enzyme.

[0012] Further, the present invention provides the method of treating various diseases related to Ca^{2+}-dependent release of arachidonic acids comprising administering to said mammal an effective amount of an antibody or inhibitor set forth above with pharmaceutically acceptable carrier.

Brief Description of the Drawings

[0013] Fig. 1 shows release of [^{3}H]AA by a calcium ionopore from human and bovine RBCs.

[0014] Fig. 2 represents PLA_2 activity of fractions from the butyl-toyopearl hydrophobic column chromatography in 1st purification process of $rPLA_2$ from bovine RBCs and porcine spleen.

[0015] Fig. 3 represents PLA_2 activity of fractions from the phenyl-5PW hydrophobic HPLC column chromatography of aforementioned fraction.

[0016] Fig. 4 represents PLA_2 activity of fractions from the DEAE-5PW HPLC column chromatography of aforementioned fraction.

[0017] Fig. 5 represents PLA_2 activity of fractions from the superose 12 gel filtration FPLC column chromatography of aforementioned fraction.

[0018] Fig. 6 presents one-dimensional SDS-PAGE of final mono-Q FPLC elutes.

[0019] Fig. 7 presents two-dimensional SDS-PAGE of final mono-Q FPLC elutes.

[0020] Fig. 8 presents western blot analysis of $rPLA_2$ active pools with anti-$cPLA_2$ antibody.

[0021] Fig. 9 presents western blot analysis of $rPLA_2$ active pools with anti-$sPLA_2$ antibody.

[0022] Fig. 10 depicts the Lineweaver-Burk plot analysis of $rPLA_2$.

[0023] Fig. 11 depicts the substrate specificity of $rPLA_2$.

[0024] Fig. 12 depicts the calcium ion dependency on activity of $rPLA_2$.

[0025] Fig. 13 depicts the pH effect on the activity of $rPLA_2$.

[0026] Fig. 14 depicts the inhibition of the $rPLA_2$ activity with DTT.

[0027] Fig. 15 depicts the inhibition of the $rPLA_2$ activity with $AACOCF_3$.

[0028] Fig. 16 depicts the inhibition of the $rPLA_2$ activity with mepacrine.

[0029] Fig. 17 depicts the inhibition of the $rPLA_2$ activity with methyl mercury chloride.

[0030] Fig. 18 presents the immunoprecipitation analysis of $rPLA_2$.

[0031] Fig. 19 presents the remained PLA_2 activity of $rPLA_2$ immunoprecipitate with the lapse of time.

[0032] Fig. 20 presents the immunoprecipitation analysis of $rPLA_2$ with human phenyl-5PW fractions.

[0033] Fig. 21 presents the PLA_2 activity of human phenyl-5PW fractions.

[0034] Fig. 22 presents the effect of EPO on $rPLA_2$ expression.

[0035] Fig. 23 presents the DAB staining of MFL cells after 0 day culture.

[0036] Fig. 24 presents the DAB staining of MFL cells after 3 days culture in the absence of EPO.

[0037] Fig. 25 presents the DAB staining of MFL cells after 3 days culture in the presence of EPO.

[0038] Fig. 26 presents the DAB staining of MFL cells after 7 days culture in the absence of EPO.

[0039] Fig. 27 presents the DAB staining of MFL cells after 7 days culture in the presence of EPO.

[0040] Fig. 28 presents the immunoprecipitation analysis with $rPLA_2$ Ab of above-mentioned 5 kinds of MFL cells.

[0041] Fig. 29 presents the immunoprecipitation analysis with $cPLA_2$ Ab of above mentioned 5 kinds of MFL cells.

[0042] Fig. 30 presents the inhibition of $rPLA_2$ and $cPLA_2$ by quinone derivatives, TP1 and EA4.

[0043] Fig. 31 presents the determination of the inhibitory pattern on $rPLA_2$ by EA4.

[0044] Fig. 32 presents AA release induced by A23187, EA4, or TP1 in human RBCs.

[0045] Fig. 33 presents AA release induced by A23187, EA4 or TP1 in bovine RBCs.

[0046] Fig. 34 presents AA release induced by A23187, EA4 or TP1 in L929 cell.

Detailed Description of the Preferred Embodiment

[0047] The present invention relates to a cytosolic 42-kDa calcium-dependent PLA_2. A bovine form of the enzyme was identified using biochemical and immunochemical studies and matrix-assisted laser desorption/ionization time-of flight (MALDI-TOF) mass spectrometric analysis. These studies have shown that the isolated and purified enzyme described herein is a novel phospholipase designated as $rPLA_2$.

[0048] Accordingly, one aspect of the present invention relates to a novel cytosolic PLA_2 enzyme isolated from RBCs. Another aspect of the present invention provides an antibody that reacts specifically with the novel enzyme and methods for generating the same. Additionally, another aspect of the present invention relates to the identification of an inhibitor of the novel enzyme.

[0049] The present invention provides methods for the preparation of the novel enzyme. Also, the present invention provides methods of treating various diseases related to the Ca^{2+}-dependent release of arachidonic acids comprising administering to said mammal an effective amount of above antibody or inhibitor with pharmaceutically acceptable carrier.

[0050] One aspect of the present invention provides a novel cytosolic $rPLA_2$ enzyme derived from RBC cytosol characterized in that the enzyme produces arachidonic acid in a calcium dependent manner, has a molecular weight of approximately 42kDa as determined by SDS-PAGE, has an isoeletric point from about 3.9 to 4.1, has a maximum activity at a pH from about 9.5 to about 10, and has its specific activity of about 5.6 nM/min/mg.

[0051] The cytosolic $rPLA_2$ enzyme described herein acts on the *sn-2* position of a phospholipid and metabolizes the phospholipid to arachidonic acid (AA) in a calcium dependent manner. The cytosolic $rPLA_2$ enzyme prefers 2-AA-GPC (1-Stearoyl-2-arachidonyl-*sn*-glycerol-3-phosphocholine) among various phospholipids as a substrate. When the enzyme metabolizes 2-AA-GPC to form AA as a substrate, the *Km* and *Vmax* value of above enzyme are approximately 13.9mM and 7.4nM/min/mg, respectively. The described enzyme has a much lower specific activity (5.6 nmol/min/mg of protein) as compared to that of $cPLA_2$ (3,800-8,630 nmol/min/mg) or $sPLA_2$ (40-1,500 nmol/min/mg).

Nevertheless, the described enzyme plays an important role in metabolic processes because RBCs have the portion of up to 99 % of the blood cell mass.

[0052] The activity of inventive enzyme is not inhibited by DTT (dithiothreitol) and mepacrine ($sPLA_2$ inhibitor) as is the $cPLA_2$ enzyme. The enzyme, however, is inhibited by $AACOCF_3$ (arachidonylfluoromethyl ketone) and activated by divalent metal cation such as Zn^{2+}, Fe^{2+}, Cu^{2+}, Sr^{2+}, Ba^{2+}, Mn^{2+}, Mg^{2+} and the like.

[0053] The described enzyme is highly expressed in murine fetal liver cells (MFL) where RBC precursor cells exist in abundance. Expression of the described enzyme, however, is not induced by EPO (erythropoietin), which is RBC hematopoietic factor that activates the expression of various gene in MFL cells. However, since the differentiation of RBC to MFL cell induced in proposition to the expression rate of the described enzyme, the described enzyme is correlated with RBC hematopoesis by a different pathway than EPO.

[0054] As described above, the described enzyme metabolizes similar substrates and shows a similar calcium-dependence and optimal pH range to conventional $cPLA_2$. The described enzyme also has a similar sensitivity to various chemical compounds or divalent metal cations as that demonstrated by the conventional $cPLA_2$ enzyme. However, there are several biochemical, immunological characteristics differences and the sensitivity to specific chemical compounds that distinguish the described enzyme from conventional $cPLA_2$. For the biochemical characteristics, the described enzyme shows quite different phenomenon with $cPLA_2$ as a result of prosecuting various column chromatography. For the immunological characteristics, the described enzyme does not react with the antibodies against $cPLA_2$ or $sPLA_2$. For the sensitivity to the chemical compound, the described enzyme is not inhibited by methyl mercury, mercuric chloride or TP1 (2-(3,5-Di-*tert*-butyl-4-hydroxyphenyl)-3-chloro-1, 4-naphthalene dione) known as conventional inhibitors of $cPLA_2$ or $sPLA_2$.

[0055] In accordance with another aspect of the present invention, there is also provided a process for preparing above-mentioned enzyme comprising the steps of subjecting the cytosolic fraction obtained by crushing RBC to butyl-Toyopearl hydrophobic column, first phenyl-5PW hydrophobic HPLC column, DEAE-5PW HPLC column, Sephacryl S-300

gel filtration column, a 2nd phenyl-5PW hydrophobic HPLC, Superose 12 gel filtration FPLC column and Mono Q FPLC column chromatography consecutively.

[0056] In accordance with another aspect of the present invention, there is also provided an $rPLA_2$ antibody effectively reacting with above-mentioned $rPLA_2$ enzyme. The antibody can bind to described bovine enzyme as well as 42kDa protein isolated from human RBC cytosol, however, the antibody does not react with $cPLA_2$ or $sPLA_2$. The antibody can be prepared by mixing the instant enzyme with an equivalent amount of adjuvant, injecting the mixture onto a mouse and collecting the serum from the mouse. For example, the active lysate of $rPLA_2$ enzyme obtained from a bovine source is concentrated to a concentration of 25μg per 0.25ml. The concentrated protein solution is mixed with the equal amount of adjuvant and then the mixture is injected into mouse at the interval ranging one week to three weeks, from three to four times, preferably four times. The immunized mouse is sacrificed and the serum was obtained.

[0057] In accordance with another aspect of the present invention, there is also provided an pharmaceutical composition comprising a therapeutically effective amount of EA4 (7-chloro-6- [4-(diethylamine)phenyl]-5,8-quinolinedione) compound and a pharmaceutically acceptable carrier. This composition has utility in treating diseases caused by disorder of hematopoietic system related to $rPLA_2$ enzyme.

[0058] In accordance with another aspect of the present invention, there is also provided a use of EA4 (7-chloro-6-[4-(diethylamine)phenyl]-5,8-quinolinedione) compound for inhibiting $rPLA_2$ enzyme. EA4 can inhibit the activity of $rPLA_2$ competitively at the inhibition constant (*Ki*) of 130 μM and inhibits the release of Ca^{2+}-dependent arachidonic acid in bovine RBC. Moreover, EA4 inhibits the activity of $cPLA_2$, which is different from conventional PLA_2 inhibitors that show activity against only one species of PLA_2.

[0059] Accordingly, the described $rPLA_2$ enzyme, antibodies with specific activity against the enzyme, and the EA4 compound can be useful to treat or prevent $rPLA_2$ related diseases and to study the physiological phenomena related to RBC such as hemostasis, thrombosis and RBC hematopoiesis, and others.

[0060] For example, when the expression, function and regulation mechanism of $rPLA_2$ enzyme are found out, the metabolic pathway of metabolites produced by $rPLA_2$ and

the origin of the diseases related to $rPLA_2$ are found out, they can help diagnosing, preventing and treating various diseases related to $rPLA_2$ by inducing a mutuation in $rPLA_2$ resulting in the reduction or inactivation of activity.

[0061] The described $rPLA_2$ enzyme can be used for the preparation of a pharmaceutical composition for the treatment of diseases caused by arachidonic acids. The pharmaceutically acceptable salt of each of the compounds may be a salt of an alkali metal, such as sodium and potassium, an alkali earth metal, such as magnesium and calcium, or ammonia or an organic base, such as, TEA, pyridine and picoline.

[0062] The described pharmaceutical formulation may be prepared in accordance with conventional procedures. For example, in preparing the formulation, the active ingredient is preferably admixed or diluted with a carrier, or enclosed within a carrier which may be in the form of a capsule, sachet or other container. When the carrier serves as a diluent, it may be a solid, semi-solid or liquid material acting as a vehicle, excipient or medium for the active ingredient. Thus, the formulation may be in the form of a tablet, pill, powder, sachet, elixir, suspension, emulsion, solution, syrup, aerosol, soft and hard gelatin capsule, sterile injectable solution, sterile packaged powder and the like.

[0063] Examples of suitable carriers, excipients, or diluents are lactose, dextrose, sucrose, sorbitol, mannitol, starches, gum acacia, alginates, gelatin, calcium phosphate, calcium silicate, cellulose, methylcellulose, microcrystalline cellulose, polyvinylpyrrolidone, water, methylhydroxybenzoates, propylhydroxybenzoates, talc, magnesium stearate and mineral oil. The formulation may additionally include fillers, anti-agglutinating agents, lubricating agents, wetting agents, flavoring agents, emulsifiers, preservatives and the like. The composition of the invention may be formulated so as to provide a quick, sustained or delayed release of the active ingredient after it is administrated to a patient, by employing any one of the procedures well known in the art.

[0064] The pharmaceutical formulation of the present invention can be administered via various routes including oral, transdermal, subcutaneous, intravenous and intramuscular introduction. For treating a human patient, a typical daily dose of the above-mentioned compounds isolated from *Regina ferula* may range from about 0.001 to 1 g/kg body weight, preferably 0.01 to 0.1 g/kg body weight, and can be administered in a single

dose or in divided doses. However, it should be understood that the amount of the active ingredient actually administered ought to be determined in light of various relevant factors including the condition to be treated, the chosen route of administration, the age, sex and body weight of the individual patient, and the severity of the patient's symptom; and, therefore, the above dose should not be intended to limit the scope of the invention in any way.

[0065] The pharmaceutical composition comprising a specific antibody or inhibitor again $rPLA_2$ can be useful to the diagnosis, prevention and treatment of the diseases caused by an excessive activity of $rPLA_2$ such as thrombosis, arterioclerosis, cerebral infarction, chronic inflammation syndrome, immunological function disorder, cancer and the like.

[0066] The pharmaceutical composition comprising inventive antibody or inhibitor can be used alone or administrated in combination with treating methods such as surgery, radiotherapy, hormonal therapy, chemical therapy and biological response modulator.

EXAMPLES

[0067] The following Examples are intended to further illustrate the present invention without limiting its scope.

Example 1

The preparation of $rPLA_2$ and the identification of its characteristics

1) Materials and Methods

[0068] Stearoyl-2-[1-^{14}C]arachidonyl-*sn*-glycerol-3-phosphocholine (2-[1-^{14}C]AA-GPC) (55.3 mCi/mmol), 1-palmitoyl-2-[1-^{14}C]palmitoyl-*sn*-glycerol-3-phosphocholine (2-[1-^{14}C]PA-GPC) (55.6 mCi/mmol), 1-palmitoyl-2-[1-^{14}C]linoleoyl-*sn*-glycerol-3-phosphocholine (2-[1-^{14}C]LA-GPC) (55.9 mCi/mmol), 1-acyl-2-[1-^{14}C]arachidonyl-*sn*-glycerol-3-phosphoethanolamine (2-[1-^{14}C]AA-GPE) (55.1 mCi/mmol) and [^{3}H]arachidonic acid (AA) (204 Ci/mmol) ([^{3}H]AA) were purchased from the radio-chemical center, Amersham Life Science Ltd. (Buckinghamshire, UK). 1-Stearoyl-2-arachidonyl-*sn*-glycerol-3-phosphocholine (2-AA-GPC), dithiothreitol (DTT), A23187, 3,3'-diaminobenzidine (DAB), methylcellulose, erythropoietin and Sepharose 4B-200 gel

filtration column were purchased from Sigma Co. (St. Louis, MO). Anti-human secretory 14 kDa $sPLA_2$ antibody was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Goat anti-rabbit- and anti-mouse-alkaline phosphatase conjugates were purchased from Santa Cruz Biotechnology Inc., Santa Cruz, CA. Group IV cytosolic PLA_2 ($cPLA_2$) was purified from porcine spleen and anti-$cPLA_2$ polyclonal antibody was generated as described above(Kim,D.K., and Bonventure, J.V., *Biochem. J.*, 294, 261-270, 1993). Group II secretory PLA_2 ($sPLA_2$) was partially purified from bovine platelets as described above (Hara, S et al., *J. biochem.*, 105, 395-399, 1989). Butyl-Toyopearl 650 M gel, preparative Phenyl-5PW, analytical Phenyl-5PW, DEAE-5PW HPLC columns were purchased from Tosoh Co. (Tokyo, Japan). Sephacryl S-300 gel filtration, Superose 12 gel filtration, PD-10 desalting (Sephadex G-25 M) and Mono Q FPLC columns, and Protein A-Sepharose CL-4B beads were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). Arachidonyl trifluoromethyl ketone ($AACOCF_3$) was obtained from Biomol (Plymouth Meeting, PA). Complete Freund's adjuvant and minimal essential medium (MEM) were obtained from Gibco BRL Life Technologies Inc (Grand Island, NY). All other chemicals were of the highest purity or molecular biology grade available from commercial sources.

2) Identification of the $rPLA_2$ activity in human and bovine RBCs.

[0069] To identify the $rPLA_2$ activity in human and bovine RBCs, the releasing activity of arachidonic acid was determined.

[0070] Human venous blood was collected in heparin (40 unit/ml) from some healthy volunteers among the Korean graduate students in our laboratory and bovine blood freshly collected in heparin (40 unit/ml) in a local slaughterhouse. After blood was centrifuged at 500x g for 20 min, the resulting supernatants of the platelet-rich plasma, the buffy coat and the leading edge of the packed RBCs were completely removed by aspiration. Sedimented RBCs, leukocytes, and platelets were re-suspended in a sterile buffer (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.12 M NaCl). This centrifugation and aspiration cycle was repeated six times taking care of removing leukocytes and platelets and the top 10% of the RBCs suspensions. Washed cell suspensions (10 ml) were subsequently depleted of residual leukocytes and platelets by filtration through a Sepharose 4B-200 column (20 x 2.5 cm) pre-equilibrated with sterile saline (0.9% w/v NaCl) as described above.

[0071] The filtered cell suspensions contained the following numbers of blood cells: for human blood; <3 x 10^5 platelets/ml, <2 x 10^4 leukocytes/ml, and 4-5 x 10^9 RBCs/ml; for bovine blood; <4 x 10^5 platelets/ml, <3 x 10^4 leukocytes/ml, and 3-5 x 10^9 RBCs/ml. Differential cell counts were measured with a Coulter counter (Becton Dickinson UK, Oxford, UK).

[0072] The Sepharose 4B-200 column-purified RBCs suspensions (approximately 1 x 10^9 cells/ml) were twice washed with serum-free MEM containing 1 mg/ml of fatty acid-free bovine serum albumin (BSA) and labeled for 1 hr with 1.5 mCi [^{3}H]AA (1mCi/ 0.1ml ethanol)/ml of the same medium. Murine L929 cells (1-2 x 10^6 cells/ml) were labeled for 6 hrs with 1.5 mCi [^{3}H]AA (1mCi/ 0.1ml ethanol)/ml of the same medium. Thereafter, cells were washed three times to remove all unincorporated [^{3}H]AA. The labeled cells were incubated in MEM containing 1 mg/ml BSA as a trap for the released [^{3}H]AA and then stimulated with vehicle (0.1μl ethanol/ml medium) or 2 μ M of calcium ionophore A23187 at 37°C. And a control group is treated with vehicle only without A23187 compound.

[0073] For analysis of [^{3}H] AA release, at each time of 0, 10, 30, 60 and 90 min after reaction between the cell and A23187, the RBCs were centrifuged as above, and each aliquot (200μl) of the supernatants for the RBCs and each aliquot (100μl) of the conditioned media for the L929 cells was transferred to 2.5ml of the scintillation counting solution and counted for radioactivity with a Packard Tri-carb liquid β-scintillation counter (Packard Instrument Co., Meriden, CT).

[0074] Since the higher value of radioactivity shows that arachidonic acid labeled with radioactive isotope is released more, above experiment was repeated to assess statistical data shown in Fig. 1.

[0075] As shown in Fig. 1, a calcium ionophore A23187 released [^{3}H]AA from the purified human and bovine RBCs in a time-dependent manner. The release of [^{3}H]AA in these cells were relatively rapid as significantly observed at 10 min and gradually increased up to 60 min, which means that there exists the calcium ion dependent $rPLA_2$ activity in human and bovine RBCs.

[0076] To identify to which class type of the $rPLA_2$ in human and bovine RBCs is belonged, the substrate specificity of $rPLA_2$ in human and bovine RBCs was determined.

[0077] The total incorporated [^{3}H]AA into the RBCs was determined by centrifuging the RBC suspensions at 10,000 x g for 1 min immediately and 1 hr after addition of [^{3}H]AA, respectively, and measuring the radioactivity of each aliquot of the supernatants. The total incorporated [^{3}H]AA into L929 cells was measured by counting the radioactivity of an aliquot (50μl) of the cell lysates obtained after washing the cells three times with 10ml of PBS and then adding 1ml of 0.5 N NaOH solution. Then, 2-[1-^{14}C]AA-GPC (55.3mCi/mmol), 2-[1-^{14}C]LA-GPC (1-palmitoyl-2-[1-^{14}C]linoleoyl-*sn*-glycerol-3-phosphocholine, 55.9 mCi/mmol), 2-[1-^{14}C]PA-GPC (1-palmitoyl-2-[1-^{14}C]palmitoyl-*sn*-glycerol-3-phosphocholine, 55.6 mCi/mmol) and 2-[1-^{14}C]AA-GPE (1-acyl-2-[1-^{14}C] arachidonyl-*sn*-glycerol-3-phosphoethanolamine, 55.1 mCi/mmol) was used as substrate respectively.

[0078] As result of above experiment, a Ca^{2+}-dependent PLA_2 activity, which preferred 2-[1-^{14}C]AA-GPC to 2-[1-^{14}C]LA-GPC and 2-[1-^{14}C]PA-GPC by 8.5- and 25.2-folds, respectively, was detected in the cytosolic fractions and hydrolyzed preferentially 2-[1-^{14}C]AA-GPE to 2-[1-^{14}C]AA-GPC by 1.7-fold.

[0079] This substrate specificity for the RBC form of PLA_2 from the cytosolic fractions suggests that this enzyme may be similar to a group IV $cPLA_2$.

3) Purification of $rPLA_2$ from bovine RBCs

[0080] To isolate $rPLA_2$ from bovine RBCs cytosol, various kinds of column chromatographic methods were subjected to purify the $rPLA_2$ protein.

[0081] Additionally, to compare the biochemical characteristics between $rPLA_2$ in bovine RBCs and common group IV $cPLA_2$, as a control group, the porcine spleen tissue abundant in $cPLA_2$ was subject to purification process in a same manner.

[0082] The packed RBCs were prepared from bovine blood (4 liters) as described above and re-suspended in buffer A (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 10 mM 2-mercaptoethanol) containing 1μg/ml leupeptin, 5 mg/ml aprotinin, 1 mM DTT and 1 mM phenylmethylsulfonyl fluoride and used as the enzyme source for purification of PLA_2. Firstly, to obtain cytosolic and membrane fractions of bovine RBCs, the resuspended packed cells were homogenized by sonicating in an ice bath at 40 W output and 40% duty cycle for 20 seconds with a sonicator (Sonics & Materials inc., Danbury, CT). The debris and unlysed

cells were removed by centrifuging the homogenates at 3,000 x g at 4°C for 30 min. After the supernatants were again centrifuged at 100,000 x g at 4°C for 2 hours, the resulting supernatants and pellets were obtained as the cytosolic and membrane fractions, respectively. For the first step, the cytosolic fractions were adjusted to 0.5 M $(NH_4)_2SO_4$, stirred at 4°C for 5 min and loaded onto a Butyl-Toyopearl hydrophobic column (15.0 cm x 5.0 cm) pre-equilibrated with buffer A containing 0.5 M $(NH_4)_2SO_4$ at a flow rate of 20ml/min. After washing with buffer A until no protein was eluted, the column-binding proteins were eluted at a flow rate of 20ml/min with a stepwise gradient of distilled water. The analysis data for determining the activities of each eluted fractions was shown in Fig. 2.

[0083] Next, a pool of the active fractions was adjusted to 0.5 M $(NH_4)_2SO_4$ and then loaded onto a preparative Phenyl-5PW hydrophobic HPLC column (21.3 mm x 15 cm) pre-equilibrated with buffer A containing 0.5 M $(NH_4)_2SO_4$ at a flow rate of 5.0ml/min. The column-binding proteins were eluted at a flow rate of a 100ml linear gradient of 0.5-0.0 M $(NH_4)_2SO_4$, and 5ml fractions were collected. The analysis data for determining the activities of each eluted fractions was shown in Fig. 3.

[0084] The active fractions were pooled and loaded onto a DEAE-5PW HPLC column (7.5 mm x 7.5 cm) pre-equilibrated with buffer A at a flow rate of 1.0ml/min. Proteins bound to the column were eluted with a 20-ml linear gradient of 0.0-1.0 M NaCl, and 1ml fractions were collected. The analysis data for determining the activities of each eluted fractions was shown in Fig. 4.

[0085] The active fractions from the DEAE-5PW column were pooled and injected onto a Sephacryl S-300 gel filtration column (30 mm x 60 cm) pre-equilibrated with buffer A containing 0.1 M NaCl. The column was eluted with the same buffer at a flow rate of 1ml/min. The active pool was continuously adjusted to 0.5 M $(NH_4)_2SO_4$ and then loaded onto an analytical Phenyl-5PW hydrophobic HPLC column (7.5 mm x 7.5 cm) pre-equilibrated with buffer A containing 0.5 M $(NH_4)_2SO_4$ at a flow rate of 1.0ml/min. The column-binding proteins were eluted at a flow rate of 1 with a 20ml linear gradient of 0.5-0.0 M $(NH_4)_2SO_4$. The fractions of the major peak activity eluted were pooled and used for further purification. The active pool was concentrated into approximately 250µl using a Centricon 10 (Amicon Co., Beverly, MA) and injected onto a Superose 12 gel filtration

FPLC column (10 mm x 30 cm) pre-equilibrated with buffer A containing 0.1 M NaCl. The column was eluted with the same buffer at a flow rate of 0.5 ml/min. 0.5 ml fractions were collected. The analysis data for determining the activities of each eluted fractions was shown in Fig. 5.

[0086] Finally, this active fractions were loaded onto a Mono Q FPLC column (5.0 mm x 5.0 cm) pre-equilibrated with buffer A adjusted to pH 8.0 at a flow rate of 1.0 ml/min. Proteins bound to the column were eluted with a 20 ml linear gradient of 0.0-1.0 M NaCl, and 1 ml fractions were collected. To monitor the amount of protein during purification of $rPLA_2$, the absorbance at 280nm (A_{280}) was measured by an UV detector. Protein concentration of each sample was measured with Bradford reagents (Bio-Rad, Hercules, CA) using BSA as a standard.

[0087] As shown in Figs. 2 to 5, the bovine RBCs cytosolic $rPLA_2$ was eluted at different fractions from all of the columns utilized differently from $cPLA_2$ and in particular, the RBCs $rPLA_2$ migrated as a molecular mass of about 40kDa comparing with the $cPLA_2$ having a molecular mass of about 60kDa.

[0088] Therefore, it is confirmed that the biochemical characteristic of bovine RBCs cytosolic $rPLA_2$ is different from that of known $cPLA_2$.

[0089] The summarized result of above column chromatographic experiment is shown in Table 1.

Table 1

Summary of purification of $rPLA_2$ from bovine RBCs

Purification step	Total protein (mg)	Total activity (pmol/min)	Yield (%)	Specific activity (pmol/min/mg protein)	Fold
S100	46,000	18,400	100.0	0.4	1
Butyl-Toyopearl	700	7,280	39.6	10.4	26
Phenyl-5PW(I)	37.50	4,095	22.3	109.2	273
DEAE-5PW	7.00	1,873	10.2	267.6	669
Sephacryl S-300	1.70	1,804	9.8	1061.2	2,653
Phenyl-5PW(II)	0.52	656	3.6	1,261.6	3,154

Superose 12	0.25	412	2.2	1,648.0	4,120
Mono Q	0.06	336	1.8	5,600.0	14,000

[0090] As can be seen from Table 1, two hydrophobic columns as initial steps typically resulted in a 273-fold purification and 22.3% yield of bovine RBC cytosolic PLA_2. A Superose 12 gel filtration FPLC column resulted in a 1.3-fold purification with an efficient yield of 62% and Mono Q anion-exchange FPLC column resulted in a 3.4-fold purification with a high yield of 81%.

[0091] To assess the purity, a portion of each fraction was analyzed on one-dimensional and two-dimensional SDS-PAGE gels, respectively. The results were shown Fig. 6 and 7 respectively.

[0092] The relative PLA_2 activity from the final step paralleled the intensity of the 42 kDa band as a single protein band (*See* Fig. 6, inset), and a single spot was observed in a two-dimensional SDS-PAGE (*See* Fig. 7), indicating that this 42 kDa band represents the RBC PLA_2, termed $rPLA_2$. MALDI-TOF mass spectrometric analysis of the single spot showed no apparent homology to any known protein.

[0093] Therefore, we confirmed that the purified $rPLA_2$ is novel protein through above experiments.

4) Immunochemical study of $rPLA_2$

[0094] To identify the immunochemical characteristics of $rPLA_2$, following experiment was subjected.

Preparation of SDS-PAGE

[0095] One-dimensional denaturing SDS-PAGE was performed on 10% polyacrylamide gels according to Laemmli's procedure in a Bio-Rad Protean II electrophoresis system. Two-dimensional gel electrophoresis was performed according to O'Farrell method (O'Farrell, P.H., *J. Biol. Chem.*, 250, 4007-4021, 1975) using the IPG-phor (Amersham Pharmacia Biotech, Uppsala, Sweden) system according to the instructions of the manufacturer. The separated proteins were stained with a PlusOne silver staining kit (Pharmacia Biotech Inc. Piscataway, NJ).

Immunochemical study

[0096] The $cPLA_2$ isolated from porcine spleen (PS-$cPLA_2$) and $sPLA_2$ isolated from bovine platelet (BP-$sPLA_2$) were adopted as comparative groups and the anti-serums for each enzyme were prepared to be subjected to immunoprecipitation process as follows.

[0097] For immunoprecipitation study, pre-immune serum (50μl) and anti-42 kDa protein antiserum (50μl) were mixed with packed Protein A-Sepharose CL-4B beads (bed volume, 25μl), respectively, and incubated overnight at 4°C. The beads were then washed six times with 1.0ml of buffer B (20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 2.0% (w/v) BSA) and incubated with an active pool (protein 8.2μg) from the Superose 12 column for the indicated times at 4°C with constant shaking. Then, the beads were pelleted by centrifuging at 1,300 x g at 4°C for 1 min, and each aliquot of the resulting supernatants was assayed for PLA_2 activity. The pellets were washed six times with buffer B containing 0.1% Tween 20 and 0.5 M NaCl,.

[0098] The washed bead was subjected to electrophoresis analysis using 10% polyacrylamide gel and then the proteins isolated from the gel were transferred to nitrocellulose membrane (Hybond™ ECL™ nitrocellulose membrane, Amersham Pharmacia UK Ltd.). The membrane was reacted with primary antibody wherein the dilution ratio of the antiserum was 1: 2000. After primary antibody reaction, the 1:2,500 dilution of goat anti-rabbit or anti-mouse-alkaline phosphatase conjugate was adopted as secondary antibody. After antibody reaction, the result was identified using a preformulated substrate kit (1-Step™ NBT/BCIP; Pierce Co., Rockford, IL) shown in Fig. 8 and 9.

[0099] As can be seen in Figs. 8 and 9, $rPLA_2$ did not react with anti-spleen $cPLA_2$ (*See* Fig. 8) and anti-$sPLA_2$ antisera (*See* Fig. 9) in immunoblotting analysis, therefore, we confirmed that the immunological characteristics of $rPLA_2$ are quietly different from those of other types of PLA_2s.

5) Enzymatic characteristics of $rPLA_2$

[0100] To identify the characteristics of $rPLA_2$, the reaction velocity with substrate, specificity for substrate, calcium ion dependence, pH dependence, PLA_2 inhibitor and the sensitivity to divalent metal ion were analyzed as follows.

[0101] The active pool from Mono Q column was desalted by using PD-10 desalting column equalized with 10ml of Tris (pH 7.5) buffer solution and was reacted with various concentrations of 2-[1-^{14}C]-AA-GPC. The result shows that the apparent *Km* value was 13.9 μM and the *Vmax* value was 7.4 nmol/min/mg of protein with 2-[1-^{14}C]AA-GPC (*See* Fig. 10).

[0102] To verify the substrate specificity of $rPLA_2$, the active pool from Mono Q column was reacted with various types of phospholipids containing AA together with $cPLA_2$ as a comparative group. Each velocity of aliquot of these PLA_2 enzymes was adjusted to 0.18-0.21nmol/10min per 45 μM of 2-[1-^{14}C]-AA-GPC and 0.9 μM each substrate was used.

[0103] The result shows that $rPLA_2$ has the high selectivity for phospholipids containing AA at the *sn*-2 position like $cPLA_2$ (*See* Fig. 11).

[0104] To verify the calcium ion effect on the activity of $rPLA_2$, after various concentrations of calcium ion were added to the active pool from Mono Q column and reacted at 37°C, for 5 min, 2-[1-^{14}C]-AA-GPC as a substrate was added and reacted for further 10 min. The comparative group, $cPLA_2$, was subjected to in a same procedure.

[0105] The result reveals that $rPLA_2$ shows calcium ion dependent activity profile similar to $cPLA_2$ (*See* Fig. 12).

[0106] To verify the pH effect on the activity of $rPLA_2$, the active pool from Mono Q column was titered in various pH and reacted 2-[1-^{14}C]-AA-GPC as a substrate was added and reacted for 10 min. The comparative group, $cPLA_2$, was subjected to in a same procedure.

[0107] The result reveals that $rPLA_2$ shows maximum activity at the pH ranging from 9.5 to 10 (*See* Fig. 13).

[0108] To verify the effect of known inhibitors on the activity of $rPLA_2$, the various concentrations of inhibitors, i.e., DTT (dithiothreitol), AACOCF3 (arachidonylfluoromethyl ketone) known as the $cPLA_2$ inhibitor, mepacrine and methyl mercury, mercuric chloride known as the $sPLA_2$ inhibitors were added to active pool from Mono Q column and reacted 2-[1-^{14}C]-AA-GPC as a substrate for 10 min. The comparative groups, $cPLA_2$ and $sPLA_2$, were subjected to in a same procedure.

[0109] The result reveals that $rPLA_2$ shows maximum activity at the pH ranging from 9.5 to 10 (*See* Fig. 13).

[0110] As shown in Fig. 14, 15 and 16, $rPLA_2$ shows similar activity to $cPLA_2$, however, $rPLA_2$ showed less effective activity than $cPLA_2$ when treated with methyl mercury (*See* Fig. 17) and showed different profile from $cPLA_2$ when treated with mercury chloride.

[0111] To verify the effect of divalent metal ions on enzyme activity, to $rPLA_2$ activity, various concentrations of divalent metal i.e., Zn^{2+}, Fe^{2+}, Cu^{2+}, Sr^{2+}, Ba^{2+}, Mn^{2+} and Mg^{2+} were added to active pool from Mono Q column and reacted 2-[1-^{14}C]-AA-GPC as a substrate for further 10 min. The comparative groups, $cPLA_2$ and $sPLA_2$, were subjected to in a same procedure.

[0112] The result reveals that activity of $rPLA_2$ is similar to that of $cPLA_2$.

[0113] Therefore, $rPLA_2$ shows similarities to $cPLA_2$ in substrate specificity, calcium ion dependence, pH dependence, PLA_2 inhibitor and the sensitivity to divalent metal ion, whereas shows a little different response for enzyme inhibitors.

Example 2

The preparation of $rPLA_2$ antibody for and the identification of $rPLA_2$ function using them.

1) The preparation of $rPLA_2$ antibody

[0114] To prepare mouse anti-42 kDa protein polyclonal antibody, the active pool obtained from the Mono Q column was concentrated using a Centri-Prep (Amicon Co., Beverly, MA) by ~5-fold and an aliquot (~25μg of protein in 0.25 ml) was mixed with the same volume of complete Freund's adjuvant and injected into a BALB/c mouse via intraperitoneal route. After boosting four times at a 3-week interval, the immunized mouse was sacrificed and the serum was obtained, and it had been used as antiserum containing the antibody for 42kDa protein for further experiments (thereinafter, 42kDa Ab)

2) The identification of the $rPLA_2$ activity for AA release using $rPLA_2$ Ab

[0115] For immunoprecipitation study, pre-immune serum (50μl) and anti-42 kDa protein antiserum (50μl) were mixed with packed Protein A-Sepharose CL-4B beads (bed volume, 25μl), respectively, and incubated overnight at 4°C. The beads were then washed six times with 1.0ml of buffer B (20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 2.0% (w/v) BSA) and incubated with an active pool (protein 8.2μg) from the Superose 12 column for the indicated

times at 4°C with constant shaking. Then, the beads were pelleted by centrifuging at 1,300 x g at 4°C for 1 min, and each aliquot of the resulting supernatants was assayed for PLA_2 activity. The pellets were washed six times with buffer B containing 0.1% Tween 20 and 0.5 M NaCl, separated on 10% SDS-PAGE and visualized by a silver staining kit. The result was shown in Fig. 18.

[0116] As shown in Fig. 18, 42kDa protein band was detected when reacted with the active pool of Superose 12 column, the density of band was increased with the reaction time of bead and antiserum, whereas the control group did not show any band excluding an IgG band commonly showed at immunoprecipitation. Therefore, it is confirmed that the antibody prepared by above example directly react with $rPLA_2$.

[0117] To measure the functions of $rPLA_2$ using inventive antibody, the supernatant obtained from above procedure was adopted to analyze the activity. PLA_2 activity was measured in an assay system (100μl) of 75 mM Tris-HCl (pH 7.5) containing 45.0 mM of 2-[1-^{14}C]AA-GPC (110,000 cpm/4.5 nmol) mixed with 2-[1-^{14}C]AA-GPC, 4% glycerol, 5 mM $CaCl_2$ and 0.2 % BSA. The result was showed in Fig. 19.

[0118] As shown in Fig. 19, when 42kDa protein band was reacted with the active pool of Superose 12 column, the density of band was remarkably decreased with the reaction time of bead and antiserum, whereas the control group did not show remarkable effect. Therefore, it is confirmed that $rPLA_2$ has PLA_2 activity considering the antibody prepared by above example reduced PLA_2 activity.

[0119] To confirm the above result repeatedly, RBC protein pool obtained from the purification procedure subjected by first phenyl-5PW column chromatography and the bead were combined and then those were separated into two parts, the precipitates and supernatant.

[0120] The immunoprecipitation method using above supernatant and precipitate were subjected according to the same method with the immunochemical study described in Example 1-(3) wherein the first antibody was used with the dilution solution (1:5000) of 42 kDa Ab. The results were shown in Figs. 20 and 21 respectively.

[0121] As shown in Fig. 20, 42kDa protein band was detected in the range of No. 15 to 18 fractions and the density was highest in No. 17 fraction. This result is consistent

with the result in Fig. 21 showed that No. 17 fraction had highest $rPLA_2$ activity. The density of 42kDa protein band was in proportion to the PLA_2 activity.

[0122] Accordingly, we confirmed that 42kDa protein was bovine RBC PLA_2.

3) The identification of the $rPLA_2$ expression using $rPLA_2$ Ab and the correlation with EPO

[0123] The $rPLA_2$ expression was verified in several cells and tissues with $rPLA_2$ Ab and whether the $rPLA_2$ expression can be induced by EPO or not was also verified.

[0124] An $rPLA_2$ purified from phenyl-5PW column, $rPLA_2$ from Mono Q column and MDCK cell (lane No. 1, 2 and 3 each in Fig. 22), various units (0U, 0.2U and 0.5U) of EPO (lane No. 4, 5 and 6 each Fig. 22) for MFL cell and L929 cells, U937 cells, brain, kidney, lung, liver and spleen tissue of rat (lane No. 7, 8, 9, 10, 11, 12 and 13 each in Fig. 22) were prepared and treated in the experiment together.

[0125] Each cell was incubated for 1 - 2 weeks at 37°C maintaining the number of the cells ($2 - 3 \times 10^6$ cells/ml) and each tissue was prepared by scarifying SD male rat.

[0126] Each cell and tissue were resuspended with Solution A containing 0.12M NaCl and then homogenized by sonicating at 4-watt output and 40% duty cycle for 20s with a sonicator and the homogenates were centrifuged at 100000xg at 4°C for 1 hour.

[0127] Each 50μg of protein was adopted and subjected to undergoing immunoprecipitation with 42kDa. The result is shown in Fig. 22.

[0128] As shown in Fig. 22, the band density for 42kDa protein did not show significant change although the treatment amount at EPO was increased.

[0129] Therefore, the result suggests that $rPLA_2$ expression is not induced by EPO.

4) The Effect of $rPLA_2$ on RBC hematopoiesis using $rPLA_2$ Ab

[0130] To verify the effect of $rPLA_2$ on RBC hematopoiesis, we confirmed the correlation between the expression of $rPLA_2$ and pseudoperoxidase activity in hemoglobinized cell. The pseudoperoxidase was the enzyme publicly used as a marker for erythroid cell.

[0131] To confirm the change of pseudoperoxidase activity in hemoglobinized cell, MFL cell was incubated as follows.

[0132] In order to obtain murine fetal liver (MFL) cells, adult male and female CD-1 mice (Dae Han Biolink Co., LTD., Eumsung-Gun, Chungbuk, Korea) underwent timed matings. At days 12-13 after mating, the female mice were killed while under ether anesthesia. According to the method of Mason-Garcia *et al.* (Mason-Garcia, M., et al., (1992) *Am. J. Physiol.* **262,** C1197-1203), the fetal livers were removed from the fetuses and gently teased free of the abdominal cavity. MFL cells were gently disaggregated by sequential passage through 18-, 21-, and 23-gauge hypodermic needles, washed twice in α-modified Eagle's minimum essential medium with glutamine (α-MEM; GIBCO, Grand Island. NY), and resuspended in 5ml α-MEM. Isolated murine fetal liver cells (1 x 10^5/ml) were plated in a mixture (DAB-mixture) containing α-MEM, 0.8% methylcellulose, 20% fetal bovine serum, 10^{-4} M mercaptoethanol, 100 U/ml of penicillin, 100 μg/ml of streptomycin and 0.2 U/ml of highly purified human recombinant EPO (specific activity >160,000 U/mg protein). For DAB staining, 1ml of the DAB-mixture was plated in each 10- x 35-mm Petri dishes and incubated under a humidified atmosphere of 95% air and 5% CO_2. After 3 or 7 days, the dishes were stained for pseudoperoxidase with DAB and hydrogen peroxide and the result is shown in Figs. 23 to 27.

[0133] As shown in Figs. 23 to 27, by 3 days culture, single cells were largely reduced (Fig. 24 and 25) and instead DAB-positive colonies were found with majority as colony-forming unit erythroid (CFU-E), which consist of 10-20 cells with morphological appearance of basophilic erythroblasts and numerous mitotic figures. Most of the colonies were stained with brown color, which showed hemogloblinized. In contrast, by 7 days of culture, few erythroid colonies could be seen (Fig. 26 and 27). There is no difference between EPO treated group (Fig. 24, 26) and EPO untreated group (Fig. 25, 27) in the cell hemoglobinization and EPO treated group reproduced more number of colonies (Fig. 25).

[0134] To verify whether above result is related to the expression of $rPLA_2$ or not, the protein obtained from above cultivated MFL cell and was subjected to immuno-precipitating method in same procedure described in Example 2-(2) using $rPLA_2$ Ab or $cPLA_2$ Ab. The result was shown in Fig. 28 and 29 respectively. The protein obtained from the cell of Fig. 23 was loaded at lane No. 1 and the cell of Fig 24, 25, 26, 27 were loaded at

Lane No. 2, 3, 4 and 5 respectively and positive control group was loaded at lane No. 6 to compare with the density of each protein band.

[0135] As shown in Fig. 28, the $rPLA_2$ of present invention was expressed by 3 days culture and was not expressed further by 7 days. This result is consistent with the cell hemoglobinization profile, which means the hematopoiesis of RBCs is induced with the activation of $rPLA_2$.

[0136] On the contrary, as shown in Fig. 29, the culture day of $rPLA_2$ increase, the expression increase. Therefore, we confirmed $cPLA_2$ do not effect on erythroid differentiation of MFL cell.

Example 3

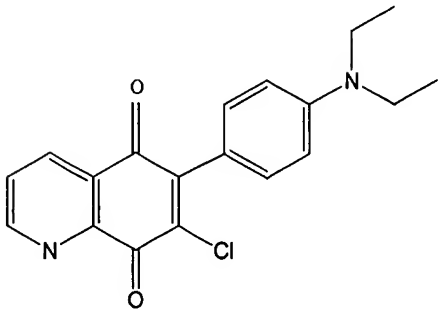
The preparation of EA4 compound, $rPLA_2$ inhibitor and the identification of $rPLA_2$ function using them.

1) The preparation of EA4 compound, $rPLA_2$ inhibitor

[0137] An $rPLA_2$ inhibitor, EA4 was prepared by following method.

[0138] 80ml of acetic acid solution containing 6.28mM 5, 8-quinolinedione and cupric acetate monohydrate respectively was mixed with the 20ml of acetic acid containing 6.28mmol diethyl aniline. And the solution was stirred for 2 hrs at room temperature and left alone for one night. The mixture solution was filtered to obtain final precipitate. The chemical formula of EA4 compound is shown in chemical formula 1.

[0139] [Chemical Formula 1]



2) The inhibitory activity of EA4 compound, $rPLA_2$ inhibitor

[0140] To identify EA4 compound inhibits $rPLA_2$ enzyme, for control group, TP1, $cPLA_2$ inhibitor was EA4 was prepared.

[0141] Prepared $rPLA_2$ and $cPLA_2$ and each inhibitor dissolved in 5μl of DMSO solution was added and reacted for 10 min at 37°C maintaining the temperature. 2-[1-^{14}C]AA-PC as a substrate was added and reacted for 10 min at 37°C maintaining the temperature. Inhibitory activities of EA4 and TP1 were estimated and showed the result in Fig. 30.

[0142] Shown in Fig. 30, EA4 inhibited both activities of $rPLA_2$ and $cPLA_2$, however TP1 inhibited that of $cPLA_2$ only.

[0143] To verify the mechanism of inhibition, Dixon plot was constructed (Fig. 31). That show that the inhibition of $rPLA_2$ by EA4 is competitive, but not uncompetitive, with an inhibition constant of Ki = 130 μM.

3) AA production by $rPLA_2$ in use of $rPLA_2$ inhibitor, EA4

[0144] To determine whether $rPLA_2$ was associated with production of AA in red blood cell, $rPLA_2$ inhibitor, EA4 was used.

[0145] According to Example 1-1), red blood cells from human and bovine, and L929 cells for control were prepared. After each type of cells were labeled with [^{3}H]AA, those were washed 3 times with MEM medium to eliminate unincorporated [^{3}H]AA. 50μM of EA4 dissolved in DMSO was added and incubated for 20 min. and then 2μM of A23187 compound also added at 37C°. For control group, 2μl of DMSO was added instead of inhibitor and A23187.

[0146] Cells were collected and centrifuged at the interval of 10min. And the radioactivity was measured by scintillation counter.

[0147] In human RBC (Fig. 32) and bovine RBC(Fig. 33), AA release by A23187 suggested in Example 1-(1) was significantly inhibited by EA4 and not inhibited by TP1.

[0148] However, in L929 cells, AA release was inhibited by both EA4 and TP1 (Fig. 34). Since there exist $cPLA_2$ in L929 cells, whose activity is inhibited by both of EA4 and TP1.

[0149] Therefore, $rPLA_2$ is correlated to AA release induced by A23187 in RBC.

[0150] As mentioned above, the novel $rPLA_2$ enzyme of present invention showed the possibility of regulator in physiological mechanism associated with RBC, having Ca^{2+}-dependent activity that alter phospholipids into arachidonic acids in bovine RBC.

[0151] The novel $rPLA_2$ antibody of the present invention, which binds 42kDa protein $rPLA_2$ specifically and the effective $rPLA_2$ inhibitor EA4 of present invention can be used in pharmaceuticals for diagnosing, preventing and treating RBC-related disorders.

[0152] While the invention has been described with respect to the above specific embodiments, it should be recognized that various modification and changes may be made to the invention by those skilled in the art that also fall within the scope of the invention as described herein and in the claims below.